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Fast form alpha-2-macroglobulin - A marker for protease activation in plasma exposed to artificial surfaces

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ABSTRACT

Objectives: Investigation of the blood compatibility requires a number of sensitive assays to quantify the activation of the blood protein cascades and cells induced by biomaterials. A global assay measuring the blood compatibility of biomaterials could be a valuable tool in such regard. In this study, we investigated whether an enzyme-linked immunosorbent assay (ELISA), that specifically measures the electrophoretic "fast form" of α_2-macroglobulin (F-α_2M), could be a sensitive and global marker for activation of calcium dependent and in-dependent proteases in plasma exposed to biomaterials *in vitro*.

Methods: A F-α_2M specific monoclonal antibody was generated and applied in an ELISA setup. Using the F-α_2M ELISA, we investigated activation of calcium dependent and in-dependent proteases by polyvinylchloride (n = 10), polytetrafluoroethylene (n = 10) and silicone (n = 10) tubings as well as glass tubes (n = 10).

Results: We found that F-α_2M is a sensitive marker for activation of both calcium dependent and in-dependent proteases. A significant difference between F-α_2M concentrations in the control sample and plasma exposed to the artificial surfaces was found ($p > 0.001$). This was observed both in the presence and absence of calcium. Furthermore, the highest F-α_2M concentration was in both cases found in plasma incubated with glass.

Conclusions: Our findings demonstrate that F-α_2M is a sensitive marker for detection of protease activation in plasma by artificial surfaces. Potentially, levels of F-α_2M could be a global marker of the blood compatibility of biomaterials.

1. Introduction

Exposure of human blood to artificial surfaces in clinical settings is frequently associated with an increased risk of thrombotic and inflammatory reactions [1–3]. These complications arise from a complex inter-play between surface induced protein adsorption, cell adhesion, and a series of blood protein cascades: the contact system, coagulation, and the complement system [4]. Protein adsorption of contact factors to artificial devices or the action of tissue factor from blood cells has been suggested to be initiators of coagulation [2,5,6]. Activation of the complement system induced by artificial surfaces is reportedly mediated through the alternative and classical pathways [7,8] and the contact system [4]. Currently, the use of biomaterials in clinical settings require administration of anticoagulants to minimize thrombotic complications. Such an approach, however, does not completely prevent the complications triggered by interaction of blood with artificial surfaces [2,4]. Furthermore, studies investigating inhibition of the complement cascade or the contact activation system, as a new approach to improve the blood compatibility of biomaterials, highlight the complexity of the interaction between blood and artificial surfaces [2,4]. Investigations of the blood compatibility and development of new biomaterials therefore call for the use of several assays measuring initiation of the contact system, coagulation, and the complement cascade as well as markers for platelet and leukocyte activation. The International Standardization Organization (ISO) 10993-4 standard describes a number of assays to study specific analytes that reveal the activation status of these biological systems. However, no global and sensitive immunoassay exists for detection of activation of proteases induced by biomaterials. Such assay could be useful in search of biomaterials with improved blood compatibility.

The broad-spectrum inhibitor, α_2-macroglobulin (α_2M), interacts with a vast number of proteinases in blood including those involved in contact activation and coagulation [9]. In human blood, α_2M circulates as a 725 kDa tetramer in a concentration of about 2.4 mg/mL [10].

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Upon interaction with a proteinase, α_2M undergoes an irreversible conformational change into a complexed or electrophoretic "fast form" (F-α_2M) [11] and exposes an otherwise hidden receptor-binding-domain (RBD) [12–14]. Based on this, we hypothesized that detection of F-α_2M could be sensitive and global approach for evaluation of the blood compatibility of biomaterials; an increase in F-α_2M levels would indicate activation of proenzymes from one or more enzymatic cascades.

In this study, we generated a F-α_2M specific antibody and applied this in an enzyme-linked immunosorbent assay (ELISA) setup to measure F-α_2M in plasma. Furthermore, we investigated whether the F-α_2M ELISA could detect activation of calcium dependent and in-dependent proteases in plasma after incubation with artificial surfaces.

2. Materials and methods

2.1. Buffers

Coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH = 9.6). PBS-TW buffer (PBS, 0.05% Tween-20, pH = 7.4). Substrate buffer (25 mM citric acid, 97 mM Na_2HPO_4, pH = 5.0). Sample dilution buffer (PBS-TW, 10 mM EDTA, 33.3 μM Phe-Pro-Arg-chloromethylketone (PPACK), pH = 7.4). TBS buffer (50 mM Tris-HCl, 150 mM NaCl, pH = 7.4 (at 37 °C)).

2.2. Plasma

A sodium citrate (0.109 M) stabilized plasma pool was obtained in-house from 20 healthy individuals (9 males, 11 females) who did not take hormone supplements. A serum pool was obtained from 22 healthy individuals (12 males, 10 females). Plasma and serum were stored at − 80 °C in 250 μL ampoules.

2.3. Reagents

Ortho-phenylenediamine (OPD) tablets were from Sigma-Aldrich Denmark ApS (Brøndby, Denmark). 3,3′,5,5′-tetramethylbenzidine (TMB) One™ ready-to-use substrate was from Kem-en-Tec Diagnostics A/S (Taastrup, Denmark). 30% H_2O_2 solution was from Merck (Darmstadt, Germany). 96-well polystyrene flat bottom MicroWell™ Maxisorp™ plates (Maxisorp plates) were from Thermo Fisher Scientific (Roskilde, Denmark). Methylamine (MA) was from Sigma.

Human native α_2M (N-α_2M) and human F-α_2M were from Sigma. Polyclonal goat anti-human α_2M IgG antibody (Cat. No. GAA2M-AP) was from Affinity Biologicals (Ancaster, Ontario, Canada). The antibody was dialyzed against PBS and subsequently biotinylated with *N*-hydroxydsuccinimide activated biotin (1 mg/6 mg antibody) for 3 h at RT. Finally, the antibody was dialyzed against PBS (biotinylated α_2M antibody). Horseradish peroxidase (HRP) conjugated streptavidin (HRP-Strp) was from Thermo Fisher Scientific. HRP conjugated rabbit anti-mouse IgG antibody (Cat. No. A9044) was from Sigma. Procoagulant phospholipid-TGT (TGT-lipid) was from Sweden). PPACK was from Haematologic Technologies Inc. (Essex Junction, Vermont, USA).

Recombinant RBD (rRBD) of human α_2M, cloned in vector pQE-30 with *E. coli* as expression system, was from Genscript (Piscataway, New Jersey, USA). The rRBD was expressed and purified as described elsewhere [15].

HiTrap™ Protein G HP 5 mL column (Protein G column) was from GE Healthcare Europe GmbH (GE Healthcare, Brøndby, Denmark).

MA-treated α_2M was prepared by incubating 1 mg/mL N-α_2M with 200 mM MA over-night (ON) at room temperature (RT). Subsequently, the sample was dialyzed against TBS buffer.

VWR Collection Silicone (Cat. No. 228-0706), Thermo Scientific Nalgene® polyvinylchloride (PVC) (Cat. No. 228-0178), and BOLA polytetrafluoroethylene (PTFE) (Cat. No. 228-0745) tubings, with a 3 mm internal diameter, were from VWR – Bie & Berntsen A/S (Søborg, Denmark). VWR Collection glass tubes, with a 10 mm external diameter and a U-bottom shape, were obtained from VWR.

2.4. F-α_2M specific monoclonal antibody

Mouse anti-human F-α_2M monoclonal antibodies were produced essentially as described previously [16]. Briefly, NMRI mice were immunized subcutaneously, three times, with 25 μg MA-treated α_2M adsorbed to $Al(OH)_3$ and mixed in a 1/1 ratio with Freund's incomplete adjuvant. A last immunization of 25 μg antigen was administrated intravenously three days prior to fusion. Spleen cells were subsequently fused with SP2/0-AG14 myeloma cells using polyenthylene glycol, and the fused cells were cultured in enriched RMPI 1640 medium. To identify hybridoma clones expressing F-α_2M specific antibodies, the culture supernatants of hybridoma cells were contra-screened using Maxisorp plates coated with either 1 μg/mL N-α_2M or F-α_2M. Wells with superior signal generation on F-α_2M coated plates were identified, and the hybridoma clones corresponding to these wells were cloned using the limited dilution method. Once single clones were obtained, the cells were grown in large scale and antibodies were purified from culture supernatant using the Protein G column. The antibodies, 16-11-2, 16-11-15, and 16-11-17, were selected for further studies to demonstrate specificity for F-α_2M.

2.4.1. Demonstration of F-α2M specificity

Recognition of rRBD by 16-11-2, 16-11-15, and 16-11-17 was investigated by ELISA. Briefly, a Maxisorp plate was coated with 4 μg/mL rRBD or 1 μg/mL N-α_2M and incubated ON at 4 °C. After a washing step, 2 μg/mL of either 16-11-2, 16-11-15, 16-11-17, or mouse anti-human tissue plasminogen activator (t-PA) antibody (nonsense antibody developed in-house), diluted in PBS-TW, were added to the wells and the plate was incubated for 60 min at RT under agitation. Following another washing step, HRP conjugated rabbit anti-mouse IgG, diluted 1:4000 in PBS-TW, was added to the wells and the plate was incubated for another 60 min at RT under agitation. Finally, the plate was washed and color development was initiated by adding 100 μL of a solution containing 0.012% H_2O_2 and 0.6 mg/mL OPD in substrate buffer. After 15 min, 100 μL 1 M H_2SO_4 was added to stop the reaction. The color development was quantified by measuring the optical density (OD) at 492 nm using OD readings at 650 nm as reference.

The electrophoretic mobility of F-α_2M and N-α_2M in presence of 16-11-17 was investigated on native PAGE. Briefly, 100 μg/mL of either F-α_2M or N-α_2M was incubated in the presence of 40 μg/mL 16-11-17 or buffer for 2 h at 4 °C in PBS. Subsequently, the samples were diluted 1:1 in 2 × native sample buffer (62.5 mM Tris-HCl, 40% glycerol, 0.01% Bromophenol Blue, pH = 6.8) (Bio-Rad) and analyzed by native PAGE using a 4–15% MiniPROTEAN® TGX™ precast gel (Bio-Rad) with TG running buffer (Bio-Rad), as described by the manufacturer, for 80 min at 150 V. The proteins in the gel were then visualized using Bio-Safe™ Coomassie G-250 stain (Bio-Rad) as instructed by the manufacturer.

2.5. Assay development

During assay development, the following setup was used; Maxisorp plates were coated with 1 μg/mL 16-11-17 by adding 100 μL of antibody solution in coating buffer to each well and incubating the plates ON at 4 °C. The plates were washed three times with PBS-TW and blocked for 40 min in PBS-TW buffer. Samples were diluted in PBS-TW (with 10 mM EDTA) and 100 μL was added to the plates and incubated for 60 min at RT under agitation. Following another washing step, 100 μL of diluted biotinylated α_2M antibody (1:2000 in PBS-TW) was added to the wells and the plates were incubated for 60 min at RT under agitation. Next, the plates were washed 3 times with PBS-TW, and 100 μL HRP-Strp, diluted 1:4000 in PBS-TW, was added to each well. The plates were incubated for 60 min at RT under agitation. Color development was initiated by adding 100 μL of a solution containing

0.012% H_2O_2 and 0.6 mg/mL OPD in substrate buffer. After 12 min, 100 μL 1 M H_2SO_4 was added to stop the reaction. The color development was quantified by measuring the OD at 492 nm using OD readings at 650 nm as reference. To calculate the concentration of F-α_2M in the samples tested, a 4-parameter nonlinear logistic (4-PL) curve was fitted to the OD measurement obtained from the calibrator wells. The fit was used to convert the measured OD values of the samples into F-α_2M concentrations. The calculated concentrations were multiplied by the initial dilution factor.

2.5.1. Spike and recovery

The citrate plasma pool was spiked with 0, 1.0, or 1.5 μg/mL F-α_2M. The level of F-α_2M in the plasma samples (diluted 1:100) were measured using the assay setup described in Section 2.5 and compared with the theoretical increase. The experiment was conducted three times with quadruple measurements. The recovery was calculated as a percentage of the mean values.

2.5.2. Parallelism

Parallelism between serial dilutions of the serum pool, F-α_2M in buffer (50 μg/mL), and the citrate plasma pool spiked with 50 μg/mL F-α_2M was studied. The samples were diluted 1:100 in sample dilution buffer and further 2-fold diluted on the plate. The dilutions were analyzed in duplicates as described in Section 2.5. The obtained data were then fitted to a 4-PL curve.

2.5.3. Sample dilution buffer

The effect of PPACK in the sample dilution buffer was studied. The citrate plasma pool and the serum pool were diluted 1:100 or 1:4000, respectively, and analyzed as described in Section 2.5 with the only difference that the samples were diluted in PBS-TW (with 10 mM EDTA) with or without 33.3 μM PPACK and incubated for 60, 120, or 180 min on the plates.

2.6. F-α_2M ELISA

Maxisorp plates were coated with 2 μg/mL 16-11-17 by adding 100 μL of antibody solution in coating buffer to each well and incubating the plates ON at 4 °C. The plates were then washed three times with PBS-TW and the wells were blocked for 120 min in PBS-TW buffer. Samples and calibrator were diluted in sample dilution buffer and 100 μL was added to the plates and incubated for 60 min at RT under agitation. Following another washing step, 100 μL of diluted biotinylated α_2M antibody (1:500 in PBS-TW) was added to the wells and the plates were incubated for 60 min at RT under agitation. The plates were washed 3 times with PBS-TW and 100 μL HRP-Strp, diluted 1:3000 in PBS-TW, was added to each well. The plates were incubated for 60 min at RT under agitation. Color development was initiated by adding 100 μL of TMB One substrate solution to each well. After 9 min, 100 μL of 0.2 M H_2SO_4 was added to each well to stop the reaction. The color development was quantified by measuring the OD at 450 nm using OD readings at 650 nm as reference. A 4-PL curve was fitted to the OD measurement obtained from the calibrator wells. The fit was used to convert the measured OD values of the samples into F-α_2M concentrations. The calculated concentrations were multiplied by the initial plasma dilution factor.

2.6.1. Freeze-thaw

Five freeze-thaw cycles were conducted of the citrate plasma pool and the citrate plasma pool spiked with 0.5 μg/mL F-α_2M (the plasma was spiked during the first cycle). A cycle was defined as follows: The sample was thawed in a water bath at 37 °C for 5 min following incubation at RT for 15 min and finally frozen again at − 80 °C for 20 min. After the five cycles, all of the samples, together with a new ampoule of plasma, were thawed and analyzed for F-α_2M as described in Section 2.6. Each sample was analyzed twice and was measured in duplicates.

2.6.2. Imprecision

The imprecision of the F-α_2M ELISA was investigated by determining the intra -and inter-assay variation of three samples with either “low”, “medium”, or “high” OD values compared to the calibrator OD values. Six series were run as described in Section 2.6. On each plate, ten replicates of the citrate plasma pool (“low”), the citrate plasma pool spiked with 0.5 μg/mL F-α_2M (“medium”) and the citrate plasma pool spiked with 1 μg/mL F-α_2M (“high”) were analyzed in duplicate. The mean values, standard deviation, and coefficient of variation (CV) were then calculated. The intra-variation is reported as mean CV of the intra-CV calculated for each plate. The inter-variation is reported as the mean CV of the inter-CV between each replicate on the six plates.

2.7. Evaluating biomaterials

The plasma compatibility of silicone, PVC, and PTFE tubings as well as glass tubes was investigated *in vitro*. The different tubings were cut in 5 cm pieces and, together with the glass tubes, warmed to 37 °C in a pre-warmed incubator for 30 min. A 100 mM $CaCl_2$ solution and a 24 μM TGT-lipid solution were prepared by diluting stock solutions in TBS-buffer. One millilitre of the $CaCl_2$ and TGT-lipid solutions as well as TBS-buffer were heated to 37 °C for 15 min in a waterbath. After 30 min, the citrate plasma pool was thawed for 10 min at 37 °C in a waterbath. For activation of calcium in-dependent proteases, plasma was mixed with 1/3 pre-warmed TBS-buffer. 150 μL of the mixture was immediately added to each of the different materials and incubated for 40 min at 37 °C. Each material was investigated 10 times. The plasma was then diluted 1:20 in PBS-TW buffer containing 10 mM EDTA and 200 μM PPACK and frozen at − 80 °C. Subsequently, the samples were analyzed as described in Section 2.6. The same procedure was followed for activation of calcium dependent proteases, but plasma was instead diluted with 1/6 part $CaCl_2$ solution (final concentration 16.7 mM) and 1/6 part TGT-lipid solution (final concentration 4 μM) and incubated for 60 min at 37 °C. Each material was investigated 10 times. The plasma was then collected from the different materials by centrifugation (4000 g) for 2 min, diluted 1:20 in PBS-TW buffer containing 10 mM EDTA and 200 μM PPACK, and frozen at − 80 °C. Subsequently, the samples were analyzed as described in Section 2.6.

3. Results

3.1. Characterization of antibody

The native PAGE (Fig. 1) demonstrated that antibody 16-11-17 recognizes human F-α_2M but not human N-α_2M. A shift in electrophoretic mobility, indicating increased molecular weight, was observed for F-α_2M in the presence of the antibody (lane 4) compared to F-α_2M alone (lane 3). No shift in molecular weight was observed when N-α_2M was incubated with 16–11-17 (lane 1 & 2).

The ELISA experiment demonstrated that antibody 16-11-17 recognizes rRBD of α_2M. Antibodies 16-11-2 and 16-11-15, on the other hand, recognize N-α_2M but not rRBD of α_2M. No signal was observed with a mouse anti-human t-PA antibody (nonsense antibody) (Table 1).

3.2. F-α_2M ELISA

Spiking of plasma with 1 μg/mL F-α_2M revealed a recovery percentage of 107% compared to the theoretical increase, whereas addition of 1.5 μg/mL F-α_2M to plasma showed a recovery percentage of 106%. Parallelism was observed between serial dilutions of purified F-α_2M in buffer, serum, and a citrate plasma pool spiked with F-α_2M. In Fig. 2, the OD values obtained at the different dilutions, together with the corresponding logistic fitted curves, are shown. The curves show a

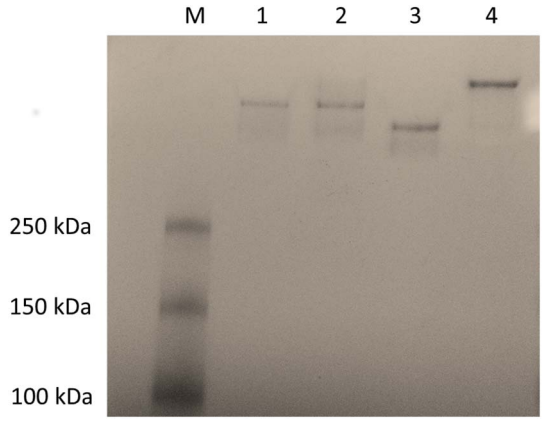

Fig. 1. Electrophoretic mobility shift assay. Native PAGE analysis of native α_2-macroglobulin (N-α_2M) and fast form α_2-macroglobulin (F-α_2M) in presence or absence of antibody 16-11-17. Lane M: molecular weight marker. Lane 1: N-α_2M, lane 2: N-α_2M + 16-11-17, lane 3: F-α_2M, lane 4: F-α_2M + 16-11-17.

Table 1
Overview of antibody specificity. The interaction between the antibodies and either recombinant receptor-binding domain (rRBD) of α_2-macroglobulin (α_2M), native α_2M (N-α_2M), or fast form α_2M (F-α_2M).

Antibody	rRBD	N-α_2M	F-α_2M
16-11-17	+	−	+
16-11-2	−	+	ND
16-11-15	−	+	ND
Nonsense antibody	−	−	ND

Interaction is indicated by "+". No interaction is indicated by "−". ND: not determined.

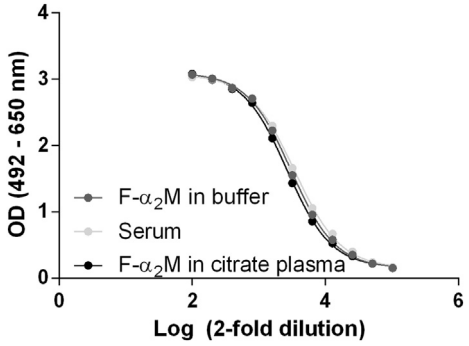

Fig. 2. Parallelism investigations. Parallelism between 2-fold dilution of buffer and citrate plasma, spiked with 50 µg/mL fast form α_2-macroglobulin, and serum.

sigmoidal shape and superimpose.

The presence of PPACK in the sample dilution buffer had a pronounced effect upon F-α_2M measurements in the citrate plasma pool but not the serum pool (Table 2). In the absence of PPACK, a considerably increase in F-α_2M levels were observed in the citrate plasma with increasing incubation time. The presence of PPACK, on the other hand, resulted in equal F-α_2M levels at the incubation times tested.

No effect from repetitive freezing and thawing the citrate plasma pool or the citrate plasma pool spiked with 0.5 µg/mL F-α_2M, upon the measured F-α_2M concentration, was observed (Fig. 3).

For the "low" OD sample, an intra-assay CV of 3.8% and an inter-assay CV of 5.7% were found. For the "medium" OD sample, an intra-assay CV of 3.8% and an inter-assay CV of 6.3% were found. For the "high" OD sample, an intra-assay CV of 3.8% and an inter-assay CV of 7.0% were found.

Table 2
Stability of fast form α_2-macroglobulin concentrations. The influence of Phe-Pro-Arg-chloromethylketone (PPACK) in the sample dilution buffer upon fast form α_2-macroglobulin (F-α_2M) concentrations in plasma and serum determined after incubation for 60, 120, and 180 min.

Incubation time (minutes)	CP (ng/mL)	CP + PPACK (ng/mL)	SP (ng/mL × 10^{-3})	SP + PPACK (ng/mL × 10^{-3})
60	1195 (708–2060)	645 (565–727)	49.4 (46.3–52.4)	49.5 (48.3–50.8)
120	1908 (934–3043)	636 (577–657)	49.0 (47.9–50.9)	47.4 (40.3–47.7)
180	3055 (1026–4755)	666 (644–687)	49.5 (47.0–52.0)	48.8 (46.1–51.4)

The results are presented as medians and range of data obtained from four experiments. CP: citrate plasma pool. SP: serum pool.

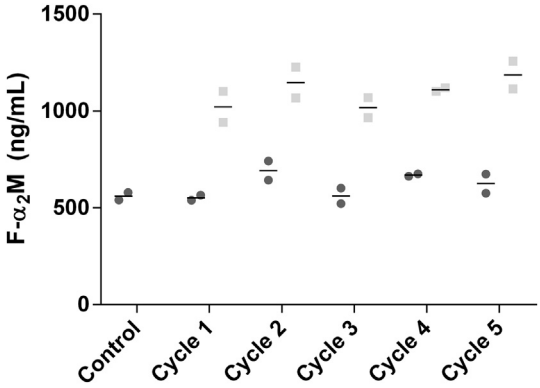

Fig. 3. The effect of freeze-thaw cycles. The concentration of fast form α_2-macroglobulin (F-α_2M) in the citrate plasma pool and the citrate plasma pool spiked with 0.5 µg/mL F-α_2M after repetitive freezing and thawing. Dark circles represent citrate plasma. Grey rectangles represent the citrate plasma pool spiked with 0.5 µg/mL F-α_2M.

3.3. Biomaterial evaluation

A significant difference in F-α_2M concentrations between the control sample (time zero) and plasma samples incubated, in the absence of calcium, in silicone, PVC, and PFTE tubings, as well as glass tubes, were observed ($p < 0.001$) (Fig. 4, Left). Furthermore, while no difference was observed between silicone, PVC, and PFTE, a 3-fold increase in F-α_2M levels was observed in plasma from glass tubes.

In the presence of calcium, F-α_2M measurements increased 20–30 fold in plasma exposed to the artificial surfaces compared to the control sample (time zero) (Fig. 4, Right). The highest increase was observed for glass (80 fold) which also was significantly higher than silicone, PVC, and PFTE tubings. Silicone tubings resulted in significantly lower F-α_2M values compared to PVC or PFTE tubings ($p < 0.05$). No significant difference was observed between PVC and PFTE tubings ($p > 0.05$).

4. Discussion

Exposure of blood to artificial surfaces initiates a complex inter-play involving surface related protein adsorption and cell adhesion as well as initiation of the contact-, coagulation-, and complement systems [4]. In clinical settings the interactions are associated with increased risk of thrombotic and inflammatory manifestations [1,3]. Investigations of the blood compatibility and development of new biomaterials currently require a range of mono-components assays to explore contributing factors. The ISO 10993-4 standard lists several assays for investigation of coagulation, the complement system, and blood cell activation. A global and sensitive marker for the blood compatibility of biomaterials could be valuable in this regard replacing or supplementing the

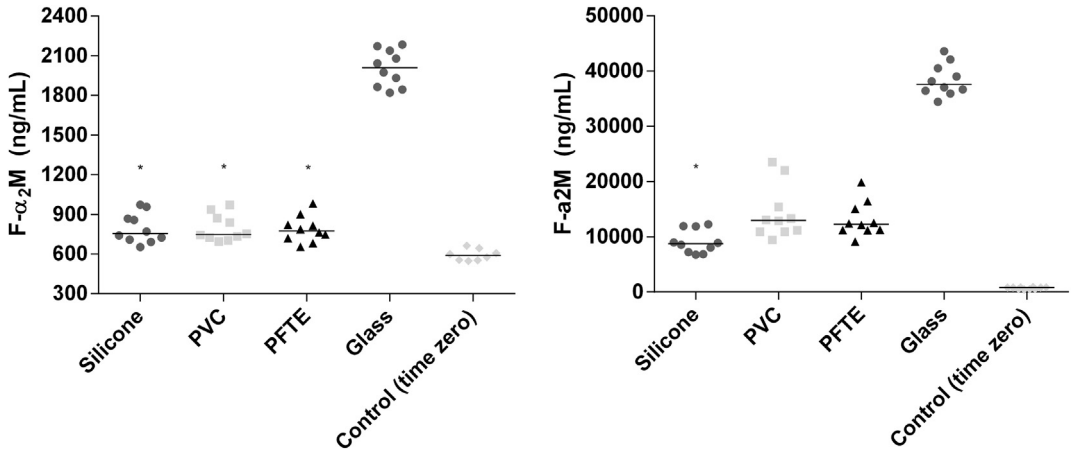

Fig. 4. The effect of artificial surfaces on the plasma concentration of fast form α_2-macroglobulin (F-α_2M) in absence and presence of calcium. Left: F-α_2M concentrations in plasma incubated without calcium in silicone (n = 10), PVC (n = 10), PFTE (n = 10) tubings, or glass tubes (n = 10) compared to the control sample. *$P < 0.001$ compared to control. Right: F-α_2M concentrations in plasma incubated with calcium in silicone (n = 10), PVC (n = 10), or PFTE (n = 10) tubings as well as glass tubes (n = 10) compared to the control sample. *$P < 0.05$ compared to PVC or PFTE. Horizontal lines indicate median levels.

proposed assays. The broad-spectrum inhibitor α_2M targets a vast number of proteases in blood [9] including those involved in the contact- and coagulation systems. In this study, we addressed whether F-α_2M could be a global and sensitive marker for activation of calcium dependent or in-dependent proteases in plasma exposed to artificial surfaces.

We generated a monoclonal antibody that reacts against F-α_2M. By native PAGE, we demonstrated that the antibody 16-11-17 specifically recognized F-α_2M but not N-α_2M. Furthermore, we showed that 16-11-17 recognizes rRBD of human α_2M which is only exposed in F-α_2M [12–14]. Using this antibody, an ELISA measuring F-α_2M was established. We observed parallelism between serial dilutions of serum, F-α_2M in buffer, and citrate plasma pool spiked with F-α_2M. Furthermore, the theoretical increase in F-α_2M levels of the spiked citrate plasma pool corresponded well with the measured increase showing comparability between results from different plasmas and buffer.

The ELISA antigen incubation step greatly affected the plasma concentration of F-α_2M which increased considerably with increasing incubation time. Addition of the broad spectrum serine protease inhibitor, PPACK, to the sample dilution buffer was necessary to prevent the increase in F-α_2M during the incubation step. This observation suggests that the increase in F-α_2M was related to pre-analytical protease activation of the sample, presumably initiated by the high binding ELISA plate, and indicates that F-α_2M is a sensitive marker for activation of proenzymes in plasma exposed to artificial surfaces.

The F-α_2M ELISA could detect protease activation in plasma incubated with silicone, PVC, and PFTE tubings (plastic materials) as well as glass tubes with or without addition of calcium. In the absence of calcium, we observed that F-α_2M concentrations were significantly higher in plasmas incubated with plastic materials or glass compared to the control sample. The highest F-α_2M levels were observed in plasma incubated with glass. The proteases of the contact system are calcium in-dependent. Furthermore, as glass is known to activate the contact system considerably [17], these findings strongly suggest that the F-α_2M ELISA can detect activation of the contact system. The findings that F-α_2M ELISA could separate the protease activation induced by plastic materials from that of the control sample further highlights that F-α_2M is a sensitive marker for protease activation. In previous studies, the contribution from the contact system has been evaluated by measuring formation of complexes between factor XIIa and complement C1-esterase inhibitor [18,19]. However, in some cases, contact activation induced by artificial surfaces could not be detected despite evidence indicating an involvement [18]. These observations suggest that sensitive assays are required to investigate contact activation induced by biomaterials.

Exposure of plasma to plastic materials and glass, in the presence of calcium, resulted in a massive increase in F-α_2M in all samples. A 20–30 fold increase in F-α_2M levels was observed from plasma samples incubated in contact with plastic materials compared to the control sample. Plasma incubated in contact with glass showed an 80 fold increase in F-α_2M levels compared to the control sample and a 2–4 fold increase compared to levels measured in plasma from plastic materials. Furthermore, the F-α_2M levels in plasma incubated with silicone tubings were significantly different from F-α_2M levels in plasma from PVC and PFTE tubings. The coagulation system is a calcium dependent and highly potent system involving several proteases [20]. The findings described above strongly suggest that F-α_2M is a sensitive marker for the initiation and propagation of the coagulation system. This correlates well with the observation that the F-α_2M level in the serum pool is almost 100 times greater than that of the citrate plasma pool.

Other blood components including platelets and leukocytes are known to be activated by medical devices [2]. Activation of neutrophils results in the release of proteases which reportedly are regulated by α_2M [21]. Activated platelets also releases compounds including coagulation factors and other proteases [22]. Although it remains to be investigated, it is likely that F-α_2M would be a marker for activation of these blood components as well.

In conclusion, our findings demonstrate that F-α_2M measurements are useful for *in vitro* investigations of the plasma compatibility of biomaterials as it is both sensitive to limited protease activation and can detect activation of calcium dependent and in-dependent proteases. F-a2M could, potentially, be a global and sensitive marker for the investigation of the blood compatibility of biomaterials.

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